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Bacteriophage-derived endolysins restore antibiotic susceptibility in β -lactam- and macrolide-resistant *Streptococcus pneumoniae* infections

Niels Vander Elst¹, Kristine Farnen¹, Lisa Knörr¹, Lotte Merlijn¹ and Federico Iovino^{1*}

Abstract

Streptococcus pneumoniae, the pneumococcus, is a cause of major illness globally. Invasive pneumococcal disease (IPD) is characterized by pneumococci invading blood (bacteremia), lungs (pneumonia), or brain and cerebrospinal fluid (meningitis). Meningitis remains an important global health concern because half of the survivors experience long-term neurological damage. The antibiotics commonly used to treat pneumococcal infections are β -lactams and macrolides, however, *S. pneumoniae* is nowadays often resistant to one or several antibiotics, therefore novel antimicrobials are needed. Here, we found that the bacteriophage-derived cpl-1 endolysin showed consistent antibacterial activity against β -lactam- and macrolide-resistant pneumococcal clinical strains grown in human blood and human cerebrospinal fluid. Exploiting synergistic and additive mechanisms, supplementation of cpl-1 to either penicillin or erythromycin, as representatives for β -lactam and macrolide antibiotics, rescued human neuronal cells from the cytotoxicity of antibiotic-resistant pneumococcal infections. Finally, systemic administration of cpl-1 supplemented to penicillin in mice infected with penicillin-resistant pneumococci successfully reduced bacteremia, and, thanks to the efficient penetration across the blood–brain barrier, abolished bacterial load in the brain, resulting in increased (89%) survival accompanied by an asymptomatic course of infection. These findings strongly suggest that cpl-1 can enhance antibiotic susceptibility in β -lactam- and macrolide-resistant *S. pneumoniae*, serving as a valuable adjunct therapy to standard-of-care antibiotics for multidrug-resistant IPD.

Keywords *Streptococcus pneumoniae*, Invasive Pneumococcal Disease, Meningitis, Antibiotic-resistance, Endolysins, Penicillin, Erythromycin, Synergy, Neuronal damage, Inflammation, New Antimicrobial Strategy

Introduction

Streptococcus pneumoniae, the pneumococcus, is a cause of major illness globally, and, because of its high incidence of resistance towards several antibiotics, was listed among the bacterial priority pathogens in 2024 by the World Health Organization (WHO) and classified as a “serious” threat by the Centers for Disease Control

and Prevention (World Health Organization 2024; Centers for Disease Control and Prevention 2024a). The most severe manifestation of infection is invasive pneumococcal disease (IPD), characterized by pneumococci invading tissues and organs, such as blood, lungs, brain and cerebrospinal fluid leading to bacteremia, pneumonia and meningitis, respectively (Centers for Disease Control and Prevention 2024b). Bacterial meningitis, in which *S. pneumoniae* is the major cause globally (van de Beek et al., 2021), is a life-threatening inflammation of the meninges caused by a bacterial infection of the brain

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and remains a major health burden globally. Up to 50% of survivors frequently suffer from permanent neurological disabilities due to neuronal damage caused by the infection (Chandran et al., 2011; Lucas et al., 2016). Bacterial meningitis during childhood causes a significant higher risk to develop long-term neurological disorders later in adult lives, such as motor and cognitive impairment, visual and hearing loss, behavioral disturbances, and structural damage to the head (Mohanty et al., 2024). Vaccination is in place as a preventive strategy for IPD, but the emergence of serotypes that circumvent coverage by the pneumococcal vaccines, known as serotype replacement, indicates the continuous need for an effective strategy against this disease (Mcintyre et al., 2012). As such, antibiotics currently constitute the most effective way to treat IPD, including pneumococcal meningitis (van de Beek, D. et al., 2016). The current standard-of-care antibiotic treatment includes β -lactam antibiotics as well as macrolides, with penicillin and erythromycin serving as representative agents of these antibiotic classes in this study.

In addition, the emergence of antimicrobial resistant strains has become alarming, with specific concerns for increasing resistance against β -lactams and macrolides for pneumococcal infections (Jesudason, T 2024; Cheraud, R. et al., 2017; Hyde, T. B. et al., 2001). To emphasize the urge for novel antimicrobials needed, the WHO announced that antimicrobial resistance (AMR) will become a major health issue due to the increasing levels reported, also for pneumococcal meningitis (Jesudason, T 2024). Simultaneously, the number of antimicrobials under development is insufficient to tackle the challenge of increasing emergence and spread of AMR (2023 Antibacterial Agents in Clinical and Preclinical Development an Overview and Analysis n.d.). To tackle AMR emergence, endolysins derived from bacteriophages have gained increasing attention in (veterinary) medicine due to (i) their rapid method of action, and (ii) high specificity for the target bacteria (Vander Elst et al., 2024; Vander Elst, N. et al., 2023, 2020; Vander Elst, N 2024; Vander Elst et al., 2018). Endolysins are peptidoglycan hydrolases that degrade the Gram-positive cell wall resulting in osmotic lysis of the bacteria (Vander Elst, N. et al., 2020; Vander Elst, N 2024; Vander Elst et al., 2018). Endolysins are one of the major classes of novel antimicrobials under development according to the WHO (Antibacterial Agents in Clinical and Preclinical Development an Overview and Analysis n.d.), with cpl-1 (a dimer) and cpl-7 s (a monomer) being previously characterized as effective endolysins against the pneumococcus (Díez-Martínez, R. et al., 2013; Doehn, J. M. et al., 2013; Entenza et al., 2005; Harhala, M. et al., 2018; Vouillamoz, J. et al., 2013; Díez-Martínez, R. et al.,

2015; Valente, L. G. et al., 2022; Grandgirard et al., 2008). Remarkably, the knowledge on how endolysins can be beneficial in case of multidrug-resistant bacterial infectious disease, in particular meningitis, is still scarce (Valente, L. G. et al., 2022; Grandgirard et al., 2008).

Through *in vitro* infection experiments using blood, cerebrospinal fluid and neuronal cells, all human-derived, and *in vivo* through our established bacteremia-derived meningitis mouse model, we provided evidence that cpl-1 endolysin enhances antibiotic susceptibility in β -lactam- and macrolide-resistant pneumococcal infections, preventing neuronal cell death, dramatically reducing bacterial load in the blood, in the heart and, importantly, completely abolishing bacterial presence in the brain thanks to cpl-1's efficient capability to cross the blood-brain barrier (BBB). Furthermore, endolysin treatment was also capable of suppressing inflammation, therefore promoting the maintenance of a safe environment for preserving normal neuronal activity. Endolysins should therefore be considered as an essential adjunctive to the current standard-of-care antibiotics to fight multidrug-resistant bacterial invasive disease.

Materials and methods

Plasmid construction and *de novo* synthesis

The cpl-1 and cpl-7 s coding sequences were codon optimized for expression in *E. coli* and chemically synthesized by Twist Bioscience (California, USA). A C-terminal hexahistidine-tag was included for purification purposes. All constructs were cloned into a pET28a(+) vector. Benchling (Biology software, 2024) was used for DNA sequence analysis and manipulations.

Plasmid transformation, protein expression and purification

For *in vitro* experiments, chemocompetent *E. coli* BL21 (DE3) (New England Biolabs) were transformed with 25 μ g (5.0 ng/ μ L) plasmid via heat shock during 45 s at 42°C after a 30 min incubation on melting ice. The transformed cells were then incubated for 1 h at 37°C in super optimal broth after a 5 min recovery on melting ice. *E. coli* cells were subsequently plated on Luria-Bertani (LB) agar (Oxoid) with the addition of 100 μ g/mL kanamycin sulfate (Gibco). One colony was picked, transferred to a culture tube with 5 mL LB and 100 μ g/mL kanamycin sulfate, and incubated at 37°C and 200 rotations per minute (rpm) during 18 h. Next, overnight cultures were diluted 1:100 in LB broth supplemented with 100 μ g/mL kanamycin sulfate and grown in Erlenmeyer flasks at 37°C and 200 rpm. When the OD_{620nm} reached 0.6 to 0.8, protein expression was induced by the addition of 0.5 mM isopropyl β -D-1-thiogalactopyranoside (Fisher Scientific) while shaking the Erlenmeyer flasks at 100

rpm at 21°C during 18 h. Subsequently, *E. coli* was pelleted and resuspended in 10 mL B-PER (Fisher Scientific) with the addition of 10 µL of 2500 U/mL DNaseI (Fisher Scientific). Bacterial suspension was then incubated on melting ice for 15 min on a shaker at 40 rpm. Next, centrifugation (4000 g; 20 min) was performed to pellet cellular debris, and the lysate was applied to a His Gravitrap column (Cytiva). This column was washed with 4.0 mL of: (i) lysis buffer (phosphate-buffered saline (PBS) with 10 mM imidazole (Carl Roth)), (ii) wash buffer (PBS with 50 mM imidazole and 1.0 M NaCl; pH 7.4) and (iii) eluted from the column in storage buffer (PBS with 500 mM imidazole, 0.5 M NaCl and 10% glycerol; pH 7.4) and kept at -80°C. After confirming correct protein expression and purification, large-scale protein production was conducted by the Protein Science Facility (PSF) at Karolinska Institutet. This involved a two-step purification process, being immobilized metal affinity chromatography followed by size-exclusion, performed on an Äkta Pure system (Cytiva). Protein sizes were verified by PSF using MALDI-TOF mass spectrometry.

For *in vivo* experiments, lipopolysaccharide (LPS)-deficient electrocompetent ClearColi BL21 (DE3) (Biosearch Technologies) were transformed with plasmid DNA by electroporation following the manufacturer's protocol. Protein expression and purification were carried out similarly to the *in vitro* experiments, with an additional step to remove residual LPS. Pierce™ high-capacity endotoxin removal spin columns (Fisher Scientific) were used, resulting in protein with LPS levels below 0.1 EU/mL

(essential for intravenous injection in mice), as measured by the Pierce™ chromogenic endotoxin quant kit (Fisher Scientific). The day before the experiment, a buffer exchange was performed to replace elution buffer with PBS using a Pierce™ protein concentrator with a 10.0 kDa molecular weight cut-off (Fisher Scientific).

Pneumococcal isolates, culture conditions and whole genome sequencing

A panel of clinically relevant pneumococcal isolates, collected from patients with IPD (bacteremia and sepsis) or meningitis, was assembled (Table 1). This panel included the reference laboratory strains *S. pneumoniae* TIGR4 (serotype 4) and D39 (serotype 2), along with 12 additional clinical isolates representing a total of 11 serotypes. Notably, the panel featured serotypes not included in the currently available pneumococcal conjugated vaccines, such as 15 A, 16 F, 35 A and 35B (Centers for Disease Control and Prevention 2024c). The panel included one penicillin-resistant serotype 35B isolate, with a MIC of 5.0 µg/mL, one erythromycin-resistant serotype 6A isolate, with a MIC of 4.0 µg/mL, and two isolates belonging to serotypes 23 F and 35B resistant to both penicillin, with MIC of, respectively, 6.0 and 2.5 µg/mL, and erythromycin, both with MIC of 2.0 µg/mL.

S. pneumoniae isolates were cultured either on blood agar plates (Karolinska Hospital, Sweden) or in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) (Karolinska Hospital, Sweden) and incubated at 37°C in a 5% CO₂ atmosphere. For whole-genome

Table 1 Pneumococcal isolates of this study. The reference laboratory strains D39 and TIGR4 as well as 12 clinical isolates derived from either invasive pneumococcal disease (IPD) or meningitis patients were tested for resistance against penicillin (MIC > 2.0 µg/mL) as well as erythromycin (MIC > 0.25 µg/mL). ^aIndicates a MIC above the EUCAST clinical breakpoint for resistance, whereas n.d. means not determined

Isolate	Serotype	Origin	MIC penicillin (µg/mL)	MIC erythromycin (µg/mL)	Piliated
D39	2	IPD	0.08	0.06	no
TIGR4	4	Meningitis	0.08	0.03	yes
AH 4,393	23 F	IPD	0.08	0.01	n.d.
AH 16,031	35 A	IPD	1.25	0.03	n.d.
AH 18,120	6 A	IPD	0.60	0.01	n.d.
AMR 6 A	6 A	Meningitis	0.15	4.00 ^a	yes
AMR 12,116	35B	IPD	2.50 ^a	2.00 ^a	yes
AMR 19,000	35B	IPD	5.00 ^a	0.06	yes
AMR 68,715	23 F	Meningitis	6.00 ^a	2.00 ^a	yes
CCUG 35,561	18 C	Meningitis	0.08	0.06	n.d.
CCUG 32,535	22 F	Meningitis	0.08	0.03	n.d.
CCUG 35,268	9 V	Meningitis	0.08	0.06	n.d.
FI 15 A	15 A	Meningitis	0.08	0.01	yes
FI 16 F	16 F	Meningitis	0.08	0.03	yes

sequencing, a single colony was selected from a plate and grown in THY for 18 h at 37°C with 5% CO₂. Genomic DNA was then extracted using the PureLink™ Genomic DNA Mini Kit (Fisher Scientific) following the manufacturer's instructions. DNA of sufficient quality was sent to Plasmidsaurus (Oregon, USA) for sequencing and genome assembly. The resulting sequencing data, in FASTA format, were submitted to the PubMLST database (<https://pubmlst.org/organisms/streptococcus-pneumoniae>) (accessed on 30 September 2024) to confirm the bacterial species and identify allelic matches. Each *S. pneumoniae* isolate was characterized by its allelic profile, defined by allele numbers at seven loci: *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*. Based on the combination of these alleles, the sequence type (ST) was determined.

Determination of the minimal inhibitory concentration and checkerboard assays

Minimum inhibitory concentrations (MICs) were determined according to EUCAST standards (EUCAST 2024). A bacterial inoculum of 5×10^5 CFU/mL was prepared in Mueller–Hinton Fastidious (MH-F) broth (Karolinska Hospital, Sweden) supplemented with 5% lysed horse blood and 20 mg/L β-NAD (Svenska LABFAB). Plates were incubated for 18 h at 37°C, and bacterial growth was assessed by measuring the OD_{620nm} values using a spectrophotometer. The MIC was defined as the lowest concentration of antimicrobial that inhibited bacterial growth. The antimicrobials tested included benzylpenicillin (Meda) with a clinical resistance breakpoint of 2.0 µg/mL and erythromycin (Fisher Scientific) with a breakpoint of 0.25 µg/mL. Additionally, the endolysins *cpl-1* and *cpl-7* were tested, although breakpoints for these agents have not yet been defined.

Checkerboard assays were conducted to evaluate potential synergy, additive, or antagonistic effects between endolysin *cpl-1* and either penicillin or erythromycin. In brief, both antimicrobials were tested individually and in combination at varying concentrations to calculate the fractional inhibitory concentration (FIC). The FIC index was used to interpret the interaction, with FIC values indicating synergy (FIC ≤ 0.5), additive effects (FIC 0.5–4.0), or antagonism (FIC > 4.0). More detailed information on the checkerboard assay can be consulted in Bellio et al. (2021).

Evaluation of the endolysin's lytic and antibacterial activity

The peptidoglycan hydrolyzing and bactericidal activity of the endolysin was quantified using turbidity reduction (TRA) and time-kill assays (TKA) detailed in Vander Elst (2024). For the TRA, a mid-log phase bacterial culture was pelleted (4000 g, 10 min), washed with PBS, resuspended, and diluted 1:1 in PBS to an OD_{620nm} of

approximately 1.0. Then, 100 µL of the bacterial suspension was mixed with an equal volume of 5.0 µM endolysin in PBS (resulting in a final concentration of 2.5 µM) or with PBS alone as a negative control. OD_{620nm} was measured kinetically every 30 s for 1 h at 37°C using a plate reader, shaking between readings. The enzymatic activity was calculated as (ΔOD_{620nm}/min)/µM, standardized for endolysins as previously described in Briers et al. (2007). The TRA was also conducted in pooled human cerebrospinal fluid (Medix Biochemica, USA) by replacing PBS with cerebrospinal fluid in the assay. For the TKA, blood obtained from healthy volunteers (Karolinska Blodcentralen, Sweden) was spiked with mid-log phase pneumococci and subsequently treated with a final concentration of 5.0 µM *cpl-1* endolysin or PBS as a negative control. After 2 h of incubation at 37°C, serial dilutions were made and plated on blood agar plates to determine the remaining CFU/mL after incubation during 18 h at 37°C and 5% CO₂.

Culture, differentiation and infection of SH-SY5Y neuronal-like cells

Human SH-SY5Y neuroblastoma cells (ATCC) were cultured as previously described by our group (Tabusi, M. et al., 2021). Briefly, cells were seeded in 12-well plates at a density of 100,000 cells/mL, differentiated for 10 to 14 days using differentiation media consisting of EMEM (ATCC) and F12 (Gibco) in 1:1 ratio, 5% fetal bovine serum (FBS) (Gibco), 1% penicillin/streptomycin (Gibco), and 10 µM retinoic acid (RA) (Bio-Techne) and incubated at 37°C with 5% CO₂. The day before infection, neuronal-like cells were put into differentiation media without antibiotics as previously described (Tabusi, M. et al., 2021) (EMEM and F12 in 1:1 ratio, 2.5% FBS, 10 µM retinoic acid). The day of the experiment, the medium was discarded, cells were washed twice with PBS and pneumococci were resuspended in differentiation media without antibiotics and added to the neurons at a multiplicity of infection (MOI) of 10. The plates were centrifuged at 50 g for 5 min, and pneumococci were allowed to interact with the cells for 90 min before treatment was initiated and incubated at 37°C with 5% CO₂. This treatment was added at a 1:1000 dilution rate.

HUVEC culture, transwell assay, and immunocytochemistry

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured in endothelial cell growth medium (PromoCell) supplemented with 15% fetal bovine serum (Gibco), 1% penicillin–streptomycin (Gibco), and 1% L-glutamine (Gibco). Transwell 6-well plates with 8.0 µm permeable polycarbonate membrane inserts (Corning) were coated with type B bovine gelatin (Sigma) and incubated overnight at 4°C prior to cell seeding. Subsequently, the

gelatin coating solution was removed, and fresh HUVEC medium was added to the lower chamber. HUVECs were seeded at a density of 200,000 cells per well onto the upper surface of the insert or onto gelatin-coated coverslips in a 6-well plate for microscopic analysis. Plates were incubated for 48 h at 37°C and 5% CO₂.

On the day of the experiment, the culture medium was replenished, and 2.0 mg/mL cpl-1 endolysin was added to the top chamber along with 1.0 mg/mL fluorescein isothiocyanate–dextran (2,000 kDa) (Sigma) as a positive control for monolayer integrity. Samples of 200 µL from the bottom chamber were collected every 30 min for up to 6 h. Fluorescence was measured using a SpectraMax M2e plate reader (Molecular Devices), while endolysin transcytosis was assessed by western blot analysis using an anti-His tag primary antibody (1:2000, Abcam, AB9108) and an anti-rabbit HRP-conjugated secondary antibody (1:5000, Invitrogen, G21234).

For immunocytochemistry, HUVECs seeded on coverslips were treated as described above. 1 h post-challenge, cells were washed three times with PBS, fixed with 4% paraformaldehyde (Histolab) for 20 min, permeabilized with 0.1% Triton X-100 (Sigma), and stained with Alexa Fluor 647-conjugated anti-His tag antibody (1:200, Abcam, AB237337) in PBS. After three additional washes with PBS, coverslips were mounted on microscopic slides using fluorescence mounting media (Dako). Coverslips were imaged on a Zeiss LSM900-Airy confocal microscope and with settings constant between respective image acquisitions.

Animal experiments and tissue collection

Five- to six-week-old male C57BL/6 J mice (JAX™, Charles River) were housed under standard conditions, with a 13:11 light/dark cycle and ad libitum access to food and water. A bacteremia-derived meningitis mouse model was employed as previously described by our group (Iovino et al., 2019). Briefly, each mouse received an intravenous injection via the tail vein of 10⁸ CFU of *S. pneumoniae* isolate AMR 12,116 suspended in PBS. Two hours post-infection, mice were administered intravenously (tail vein) with 100 µL PBS (placebo), 100 mg/kg benzylpenicillin, 40 mg/kg of endolysin cpl-1, or a combination of both. The mice were monitored every 3 h for clinical signs, which were scored based on Karolinska Institutet's veterinary protocol for assessing rodent welfare. Once an individual score or cumulative score reached 0.4 (considering 0.0 the clinical score of a healthy mouse), the animals were anesthetized using isoflurane, 100 µL of blood was obtained and the mouse perfused via the left ventricle with PBS, spleen, heart and brain were collected. For the assessment of BBB penetration of cpl-1 endolysin, mice were intravenously injected with 40 mg/

kg of cpl-1 in 100 µL PBS and euthanized after 1 h. Next, harvested organs were placed onto a 40 µm cell strainer (Corning Falcon) with the addition of 1 mL sterile PBS and crushed through the strainer with the plunger of a sterile syringe. The homogenates obtained were serially diluted, plated on blood agar plates, and incubated for 18 h at 37°C and 5% CO₂. The CFUs were counted and calculated back to CFU/mL the subsequent day. The remaining homogenates were immediately prepared for storage by adding 1% proteinase inhibitor cocktail (Fisher Scientific), allowed to rest at 4°C for 15 min, and stored at –20°C for further analyses.

ELISA for IL-6 and TNF-α on mouse tissue

Upon thawing homogenates on ice, lysates were obtained after centrifugation (13,000 g, 3 min), and protein concentration thereof determined on 1:10 dilutions by bicinchoninic acid assay (BCA) (Fisher Scientific). Standardization was done by dilution in PBS to equal protein concentrations. Quantification of IL-6 and TNF-α was performed by ELISA (Bio-Techne) according to the manufacturer's protocol, loading 100 µg of protein into each well, in duplicate.

Immunofluorescent staining of mouse tissue, microscopy analysis and signal quantification

Harvested brains were placed in 4% paraformaldehyde (Histolab) and post-fixed overnight at 4°C, placed in a 30% sucrose solution for a minimum of 48 h, and subsequently cut into coronal sections of 30 µm on a microtome (Leica SM2000). These sections were frozen in a cryoprotective solution (30% sucrose and 2% DMSO in PBS) and stored at –20°C until further processing. Prior to staining, the sections were washed in PBS, blocked with 5% goat serum (Gibco) and permeabilized with 0.3% Triton X-100 (Sigma) for 1 h at room temperature, and subsequently stained for 2 h at room temperature with Alexa Fluor 647 conjugated anti-His tag antibody (1:200, Abcam, AB237337), for the detection of cpl-1 endolysin, and Alexa Fluor 488 conjugated lectin (1:250, VectorLabs) for the detection of the BBB vasculature (Tabusi, M. et al., 2021). Next, sections were washed in PBS and mounted with ProLong diamond mountant (Invitrogen). For staining with unconjugated antibodies, following the blocking in 5% goat serum (Gibco), the sections were stained with primary antibodies overnight at 4°C. After washing with PBS, the sections were incubated with secondary antibodies for 2 h at room temperature; nuclear staining was done by adding DAPI (Abcam) for 10 min. The primary antibodies used in this study were: Iba1 (Abcam, 1:1000), NeuN (Merck, 1:250), Caspase-3 (Invitrogen, 1:500). Secondary antibodies used were Alexa Fluor conjugated anti-immunoglobulin at 1:1000

dilution (IgG Alexa Fluor 594, 488). Three tissue sections for each mouse per staining were imaged using a Mica Microhub Imaging System (Leica) or a Zeiss LSM900-Airy confocal microscope with 20 × or 63 × magnification of a 20 μm z-stack with settings kept constant between respective image acquisition used for quantification. ImageJ (Fiji) was used to analyze the confocal images. Iba1 mean intensity was measured using the “measure” plug-in. The number of NeuN positive cells was quantified by thresholding to remove noise, and then the plug-in “analyze particles” were used with a cut-off value of 10 to count all cells. Caspase-3 bright cells were counted manually.

Statistical analysis

Data were analyzed using GraphPad Prism (version 10.1.2) to calculate p values and determine statistically significant differences ($p < 0.05$). Data were analyzed as normally distributed datasets unless otherwise specified, confirmed by performing an Anderson–Darling test ($n > 6$) or by quantile–quantile plotting the residuals ($n \leq 6$). Regarding pro-inflammatory cytokines, outlier values were detected using the ROUT method with Q set to 5% and removed from the dataset. Two groups were compared with two-tailed, (un)paired t tests, and multiple groups with analysis of the variance (ANOVA) and a Bonferroni post hoc test. Kaplan–Meier curve was analyzed by means of a log-rank (Mantel–Cox) test.

Results

The cpl-1 and cpl-7 s endolysins exhibit antibacterial activity against antibiotic-resistant *S. pneumoniae* clinical isolates

All pneumococcal clinical isolates, including penicillin- and erythromycin- resistant isolates, were challenged with the cpl-1 and cpl-7 s endolysins, and, as a result, all isolates were sensitive to the action of both endolysins (Fig. 1A, Supplementary Fig. S1). More specifically, a biochemical characterization was done through TRA which revealed that the cpl-1 compared to the cpl-7 s endolysin hydrolyzed the peptidoglycan of these isolates significantly faster ($(\Delta OD_{620nm}/min)/\mu M$; $p < 0.001$) and to a higher extent (ΔOD_{620nm} ; $p = 0.066$) (Fig. 1B and C). These findings were complemented with MIC determination, following the EUCAST guidelines, which showed that MICs for cpl-1 were significantly lower than those observed for cpl-7 s (Fig. 1D). Overall, it became evident that cpl-1 consistently outperformed cpl-7 s, leading to the decision to proceed exclusively with the cpl-1 endolysin in subsequent assays.

The cpl-1 endolysin retains its bacteriolytic activity against antibiotic-resistant pneumococcal isolates in human blood and cerebrospinal fluid

To mimic the human pathophysiological conditions during bacteremia, sepsis and meningitis, the activity of the cpl-1 endolysin was evaluated against the penicillin-resistant isolate AMR 19,000, and the multidrug penicillin- and erythromycin-resistant isolates AMR 12,116

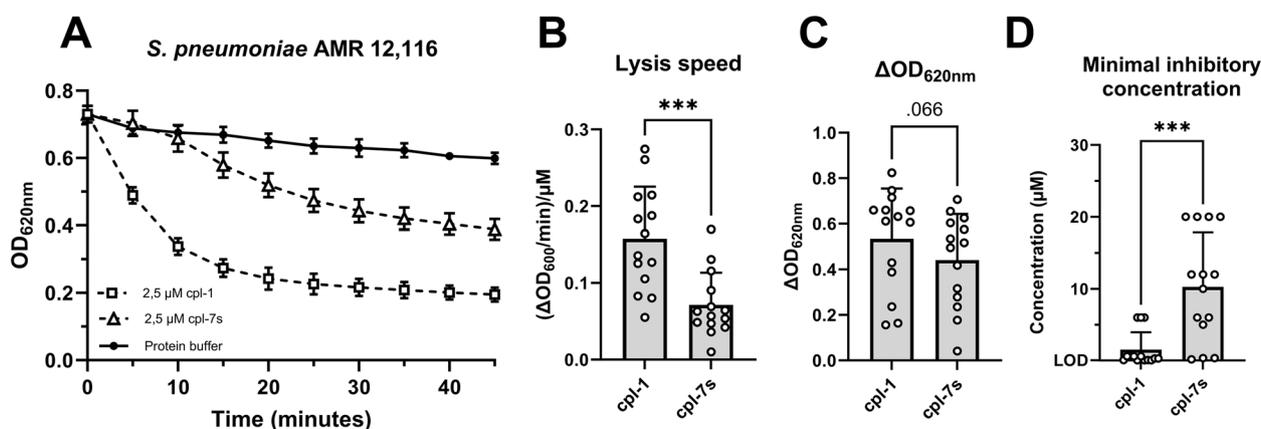


Fig. 1 Antibacterial activity of cpl-1 and cpl-7s endolysins against antibiotic-resistant pneumococcal clinical isolates. **A** cpl-1 and cpl-7s endolysins at 2.5 μM concentration displayed a strong antimicrobial activity towards the pneumococcal isolate AMR 12,116 in turbidity reduction assays. **B** and **C** Lysis speed (**B**) and ΔOD_{620nm} (**C**) analysis showed that cpl-1 hydrolyzed the bacterial peptidoglycan of all the isolates significantly faster and to a higher extent than cpl-7s. **D** Determination of minimal inhibitory concentrations (MICs) further confirmed that MICs for cpl-1 were significantly ($p < 0.001$) lower than those observed for cpl-7s for all pneumococcal clinical isolates tested. In A, data points show the mean \pm standard deviation of three biological replicates ($n = 3$) every 5 min. In B and C, each dot represents one pneumococcal clinical isolate ($n = 1$; 14 in total), and bars show the mean \pm standard deviation; Lysis speed and ΔOD_{620nm} were analyzed by means of a paired two-tailed t-test, whereas MICs were analyzed by a paired, two-tailed Wilcoxon test (MIC data are not normally distributed); LOD indicates the limit of detection (0.04 μM); * indicates $p < 0.05$, and *** indicates $p < 0.001$

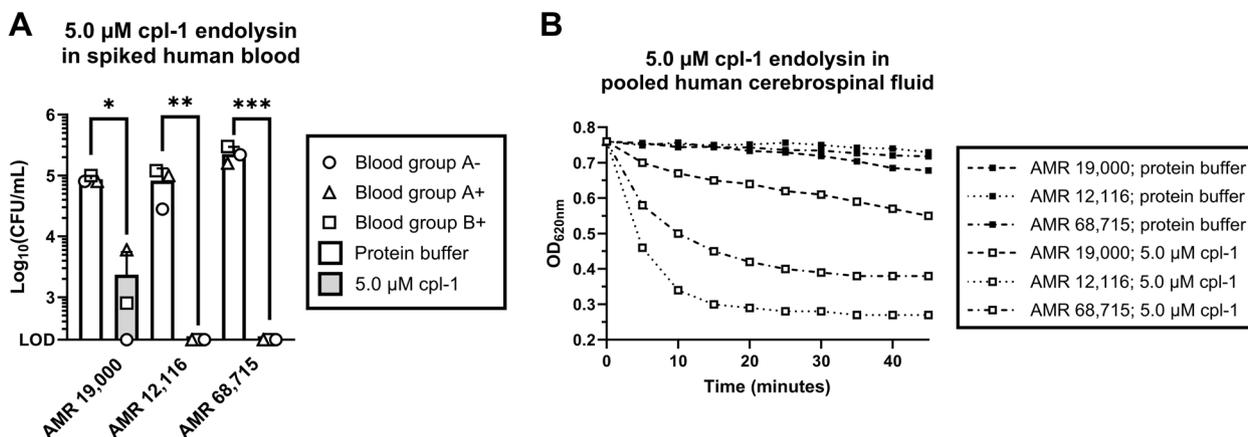


Fig. 2 Cpl-1 endolysin retains its antibacterial activity against antibiotic-resistant pneumococcal isolates in human blood and human cerebrospinal fluid. **A** Blood obtained from healthy volunteers was spiked with penicillin-resistant AMR 19,000, or penicillin- and erythromycin-resistant AMR 12,116 and AMR 68,715, which was subsequently treated with 5.0 μM endolysin cpl-1 or protein buffer as a negative control. **B** Turbidity reduction assay with the same isolates in pooled human cerebrospinal fluid. In **A**, bars show the mean ± standard deviation of three biological replicates ($n = 3$). In **B**, datapoints show one biological replicate ($n = 1$) every 5 min. $\text{Log}_{10}(\text{CFU/mL})$ were analyzed by means of a two-tailed, paired t-test; LOD indicates the limit of detection (200 CFU/mL); * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$

and AMR 68,715 in blood obtained from healthy volunteers (blood groups A-, A+ and B+) and in pooled human cerebrospinal fluid. Cpl-1 endolysin significantly reduced the bacterial load in human blood by $1.83 \pm 0.61 \log_{10} \text{CFU/mL}$ ($p < 0.05$) for AMR 19,000, and to the limit of detection (i.e., 200 CFU/mL) by 2.52 ± 0.28 and $3.04 \pm 0.11 \log_{10} \text{CFU/mL}$ ($p < 0.001$ for both) for AMR 12,116 and AMR 68,715, respectively (Fig. 2A). In pooled human cerebrospinal fluid, the lytic activity of the cpl-1 endolysin was retained as peptidoglycan hydrolysis was observed by means of TRA (Fig. 2B).

The cpl-1 endolysin lowers the MIC of antibiotic-resistant isolates below the clinical breakpoints by synergizing with penicillin, and through additive effects with erythromycin

To evaluate the impact of endolysin treatment as adjunct to standard-of-care antibiotics in the case of antibiotic resistance, checkerboard assays were conducted to assess potential synergistic, additive, or antagonistic interactions between penicillin, erythromycin, and the cpl-1 endolysin. The fractional inhibitory concentration (FIC) calculated from these checkerboard assays was consistently ≤ 0.50 for penicillin and endolysin cpl-1, which indicates a synergistic effect of cpl-1 towards penicillin (Table 2). Even though synergy was not observed for erythromycin and endolysin cpl-1, the FIC values were between 0.50 and 4.00, still indicating that important additive effects of cpl-1 are present towards erythromycin (Table 3). A remarkable finding for both antibiotics was that supplementation with endolysin cpl-1 substantially lowered the MIC values of the antibiotics very frequently

below the clinical breakpoints for resistance (Tables 2

Table 2 Checkerboard assays between penicillin and the cpl-1 endolysin with the penicillin-resistant pneumococcal isolates. The table indicates minimal inhibitory concentrations observed for penicillin, endolysin cpl-1 or the different combinations thereof. FIC is the fractional inhibitory concentration, which indicates synergy if values are ≤ 0.50 . The EUCAST breakpoint for penicillin resistance is set at 2.00 μg/mL

	AMR 12,116	AMR 19,000	AMR 68,715
Cpl-1	5.00 μM	2.50 μM	2.50 μM
Penicillin	4.00 μg/mL	4.00 μg/mL	16.00 μg/mL
Penicillin + cpl-1	0.50 μg/mL	1.00 μg/mL	1.00 μg/mL
Cpl-1 + penicillin	0.62 μM	0.62 μM	0.62 μM
FIC	0.25	0.50	0.31

Table 3 Checkerboard assays between erythromycin and the cpl-1 endolysin with the erythromycin-resistant pneumococcal isolates. The table indicates minimal inhibitory concentrations observed for erythromycin, endolysin cpl-1 or the different combinations thereof. FIC is the fractional inhibitory concentration, which indicates additive effects if values are between 0.50 and 4.00. The EUCAST breakpoint for erythromycin resistance is set at 0.25 μg/mL

	AMR 6A	AMR 12,116	AMR 68,715
Cpl-1	20.00 μM	5.00 μM	0.31 μM
Erythromycin	4.00 μg/mL	0.50 μg/mL	0.50 μg/mL
Erythromycin + cpl-1	0.50 μg/mL	0.06 μg/mL	0.01 μg/mL
Cpl-1 + erythromycin	20.00 μM	5.00 μM	0.31 μM
FIC	1.125	1.125	1.032

and 3). Of great clinical importance, the MIC values for penicillin (4.00, 4.00 and 16.00 µg/mL) were lowered 8-, 4- and 16-fold (to 0.50, 1.00 and 1.00 µg/mL) for AMR 12,116, AMR 19,000 and AMR 68,715, respectively, and thus all were consistently below the 2.00 µg/mL breakpoint (Table 2). For erythromycin, MIC values (4.00, 0.50 and 0.50 µg/mL) were lowered 8-, 8- and 32-fold (to 0.50, 0.06 and 0.01 µg/mL) for AMR 6 A, AMR 12,116 and AMR 68,715, respectively, and were below the 0.25 µg/mL breakpoint for the two latter isolates (Table 3). This strongly suggests that cpl-1 could serve as a supplemental therapy alongside standard-of-care antibiotics, potentially restoring susceptibility in antibiotic-resistant pneumococci.

Supplementation of cpl-1 endolysin to penicillin or erythromycin treatment reduces bacterial growth and mitigates cytotoxicity in human neuronal cells infected with antibiotic-resistant pneumococcal isolates

To provide preclinical proof-of-concept, human neuronal cells were infected with the penicillin- or erythromycin-resistant pneumococcal clinical isolates and subjected to various treatments that were initiated 90 min post-infection, while monitoring bacterial growth and neuronal cell

death (neuronal cytotoxicity) up to 6 h. These treatments included: (i) penicillin or erythromycin at their clinical resistance breakpoints 2.00 µg/mL and 0.25 µg/mL, respectively, (ii) a single dosage of 2.5 µM cpl-1 endolysin, (iii) three serial dosages of 2.5 µM cpl-1 endolysin, or (iv) an “antibiotic + endolysin” combination strategy following the same dosage. An untreated control was included for comparison.

As expected, under penicillin treatment, a similar increase of bacterial growth and neuronal cytotoxicity was observed to that of the untreated control across all isolates due to penicillin resistance (Fig. 3A). It was striking that, in the case of the AMR 68,715 isolate, a single dose of endolysin successfully eliminated all pneumococci and prevented the rise of neuronal cell death, making multiple dosing or an antibiotic combination strategy unnecessary for this isolate specifically (Fig. 3A). The clinical isolates AMR 12,116 and AMR 19,000 were less susceptible to the cpl-1 endolysin, with a single cpl-1 dose leading to an increase of bacterial regrowth and neuronal cytotoxicity, especially for AMR 19,000. Nevertheless, administering endolysin three consecutive times still showed a discrete improvement compared to a single dose. Remarkably, for all penicillin-resistant isolates,

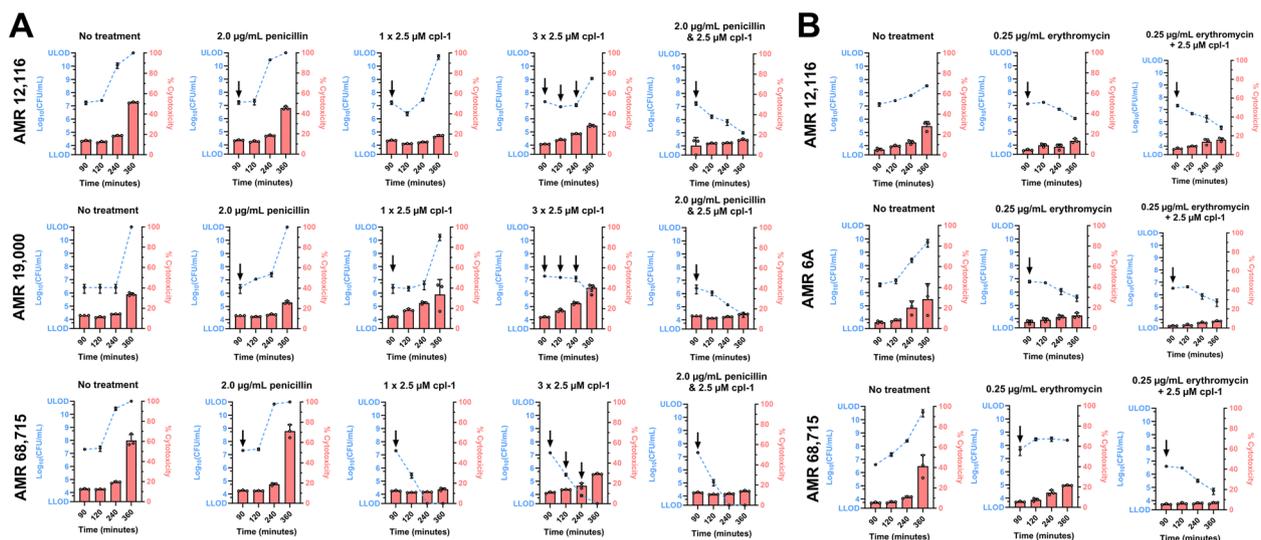


Fig. 3 Supplementation of cpl-1 endolysin to penicillin or erythromycin protects human neuronal cells from cytotoxic effect of penicillin- and erythromycin-resistant pneumococcal infections. **A** Penicillin and cpl-1 endolysin treatments were evaluated separately, including a multiple dosing strategy for the cpl-1 endolysin, and together as a combination in human neuronal cells infected with the penicillin-resistant pneumococcal clinical isolates AMR 12,116, AMR 19,000, and AMR 68,715. **B** Erythromycin treatment was evaluated as a stand-alone treatment, and together with endolysin cpl-1 as a combination, in human neuronal cells infected with the erythromycin-resistant pneumococcal clinical isolates AMR 12,116, AMR 6 A, and AMR 68,715. In both A and B, untreated controls were included for comparison; the blue dotted lines represent bacterial growth in log₁₀(CFU/mL) plotted on the left y-axis, with data points at 90-, 120-, 240-, and 360-min post-infection showing the mean ± standard deviation of three biological replicates (n = 3). The red bars depict the percentage of cytotoxicity in human neuronal cells at these same time points, plotted on the right y-axis, showing three biological replicates (n = 3; corresponding to three white circles), alongside bars indicating the mean and an error bar indicating the standard deviation. 2.0 and 0.25 µg/mL are the clinical breakpoints for penicillin and erythromycin resistance, respectively. The black arrow indicates administration of treatment. LLOD and ULOD indicate the lower and upper limit of detection of 200 and 10¹¹ CFU/mL, respectively

combining cpl-1 endolysin with penicillin, exploiting their previously identified synergy, resulting in a dramatic reduction of bacterial growth and nearly completely mitigated neuronal cytotoxicity (Fig. 3A).

In the case of erythromycin treatment, bacterial growth did not increase to the same extent as in the untreated controls, despite the isolates being previously identified as erythromycin-resistant through MIC determination. Nevertheless, the combination of cpl-1 endolysin and erythromycin was found to be the most effective in reducing bacterial growth and further decreasing neuronal cytotoxicity (Fig. 3B).

Overall, while endolysins alone may not always be effective, even when administered in multiple doses, due to their short half-life and the capability of the surviving bacteria to regrow (Vander Elst, N 2024; Grandgirard et al., 2008), the addition of cpl-1 to a standard-of-care antibiotic appears to be a very promising therapeutic strategy against antibiotic-resistant bacterial infections, especially in case of meningitis due to the great capability of cpl-1 to protect neurons and prevent neuronal cell death.

The cpl-1 endolysin crosses the blood brain barrier

Before *in vivo* studies were performed to deliver or pre-clinical proof-of-concept, we first assessed the ability of the cpl-1 endolysin to cross the blood–brain barrier (BBB) and eliminate pneumococci in the neuronal parenchyma. Using a transwell system with Human Umbilical Vein Endothelial Cells (HUVECs), we detected intact cpl-1 endolysin in the bottom chamber within 30 min, while FITC-dextran remained in the upper chamber (Fig. 4A, Supplementary Fig. S2 A, B). Confocal microscopy confirmed cpl-1 uptake by HUVECs 1 h post-treatment (Fig. 4B, Supplementary Fig. S2 C), indicating successful endothelial layer penetration by the cpl-1 endolysin.

For *in vivo/ex vivo* validation, confocal microscopy analysis using brain sections from mice intravenously treated with cpl-1 (or PBS) revealed cpl-1 presence in the microvasculature and within the parenchyma, absent in PBS-injected controls (Fig. 4C, Supplementary Fig. S3). These findings confirm that systemically administered cpl-1 endolysin efficiently crosses the BBB *in vivo*.

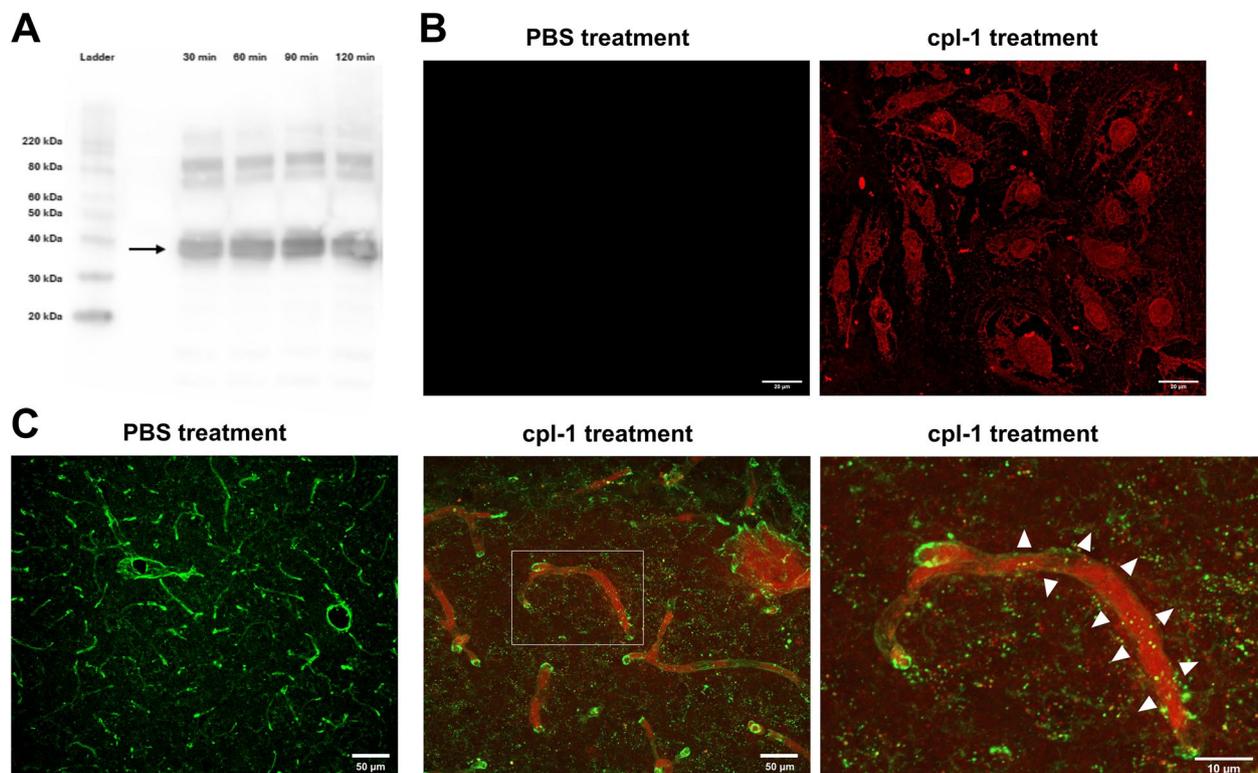


Fig. 4 Endolysin cpl-1 crosses the blood–brain barrier (BBB). **A** Western blot analysis of cpl-1 endolysin (detected using an anti-His tag antibody) in samples collected from the lower chamber of a transwell system with a HUVEC monolayer at 30, 60, 90 and 120 min. The arrow indicates the band corresponding to cpl-1 endolysin (40.3 kDa). **B** HUVECs were treated with either phosphate-buffered saline or cpl-1 endolysin, fixed 1 h post-treatment, and intracellularly stained for cpl-1 endolysin (red). Scale bars represent 20.0 μ m. **C** Confocal images display representative merged z-stacks of brain tissue sections stained for brain microvasculature (green) and cpl-1 endolysin (red); brain tissue area within white borders was enlarged to highlight the diffusion (white arrowheads) of the cpl-1 red signal from the vasculature into the brain parenchyma. The scale bars represent either 10.0 or 50.0 μ m, as indicated

Supplementation of cpl-1 endolysin to penicillin successfully treats penicillin-resistant invasive pneumococcal disease *in vivo*, including pneumococcal meningitis

We then decided to further evaluate the synergistic combination of the cpl-1 endolysin with penicillin in our established bacteremia-derived meningitis mouse model alongside placebo (i.e., PBS), endolysin stand-alone and antibiotic stand-alone treatment. Notably, the AMR 12,116 pneumococcal isolate stands out as particularly interesting given that Iovino et al. (2021) previously demonstrated that the piliated 35B serotypes usually belongs to the ST-558 pathotype and is a frequent cause of IPD in humans (Iovino et al., 2020). Furthermore, the 35B serotype is not included in the current pneumococcal vaccines and frequently has reduced penicillin susceptibility (Olarie, L. et al., 2017).

Within 13 h, all infected mice in the placebo group developed symptoms indicative of IPD and reached experimental endpoints (Fig. 5A and B). Similarly, all penicillin-treated mice succumbed within 21 h, also reaching experimental endpoint of disease. In contrast, mice treated with the cpl-1 endolysin showed a slightly improved probability of survival, most likely due to the antibacterial activity of cpl-1 towards penicillin-resistant pneumococci, which were not equally affected by the penicillin treatment; however, due to the short half-life of the cpl-1 endolysin, the antibacterial effect was only temporary, and, although the survival of this group was longer than animals treated with penicillin, most of the mice still reached the experimental endpoint within 27 h (Fig. 5A and B). On the other hand, all mice treated with the combination strategy remained asymptomatic throughout the first 21 h, displaying a significantly increased probability of survival compared to the other groups, achieving 100% survival up to 25 h post-treatment, and 89% survival at the end of the experiment (Fig. 5A and B). Notably, this reduced survival rate was due to only one mouse developing symptoms at the very end of the experiment. The group that received the combination treatment had the highest reduction of bacterial load in the blood, heart and spleen compared to all other groups (Fig. 5C and Supplementary Fig. S4); remarkably, the combination treatment completely eliminated bacteria in the brain (Fig. 5C).

Along with this significant reduction of bacterial load, the impact of antimicrobial treatment on systemic and neuroinflammation was evaluated by quantifying levels of TNF- α and IL-6 in the heart, spleen and brain, with IL-6 being an inflammatory cytokine often used as clinical marker in patients with bacterial sepsis (Yang, X. et al., 2023; Song, J. et al., 2019; Rincón-López, E. M. et al., 2021) (Fig. 5E and Supplementary Fig. S4).

Remarkably, IL-6 levels were the lowest for the combination treatment in the heart, spleen, and brain compared to all other groups. Interestingly, endolysin stand-alone treatment increased microglial activation (Supplementary Fig. S5 A, B), which we reason to be a consequence of the bactericidal effect of endolysins. To assess if the neuronal protection observed *in vitro* also extended to our mouse model *in vivo*, we assessed caspase-3-dependent apoptosis in the cortex. However, no significant differences were observed for caspase-3 bright neurons nor differences in the total number of neuronal cells between treatment groups (Supplementary Fig. S5 C-E). Taken together, these observations demonstrated that the supplementation of the cpl-1 endolysin to penicillin dramatically reduced bacterial burden and cytokines released during systemic antibiotic-resistant infection.

Discussion

IPD is without doubt a major cause of morbidity and mortality globally (European Centre for Disease Prevention and Control 2023). *S. pneumoniae* is also the leading cause of bacterial meningitis worldwide, frequently affecting children, with high risk of mortality or long-term neurological sequelae upon survival (Chandran et al., 2011; Lucas et al., 2016; Mohanty, S. et al., 2024; Kim, K.S 2010). Bacterial meningitis is treated with antibiotics, but even β -lactams, which are frequently used for streptococcal infections, have very low capability to penetrate the BBB and reaching the brain (Nau et al., 2010). Furthermore, even in case of BBB penetration, antibiotic-resistance is a constant threat to face in clinics (Hameed et al., 2010). Moreover, the rise of β -lactam- and macrolide-resistant strains is worrying (Jesudason, T. 2024; Cherazard, R. et al., 2017; Hyde, T. B. et al., 2001). Endolysins, such as cpl-1 and cpl-7s, have been previously characterized for their antibacterial activity (Valente, L. G. et al., 2022; Grandgirard et al., 2008), but fundamental proofs of their efficacy using human-derived tissue, human cells and animal models for meningitis were still missing. In this study, we delivered important preclinical proof-of-concept evidence regarding the therapeutic potential of the cpl-1 endolysin for treating invasive pneumococcal disease and meningitis, protecting human neuronal cells from the cytotoxic effect of the infection, and dampening inflammation, thanks to its ability to enhance antibiotic susceptibility in resistant infections, here demonstrated for penicillin and erythromycin as representatives for β -lactam and macrolide antibiotics, respectively.

Firstly, we demonstrated that all pneumococcal clinical isolates used in this study were sensitive to endolysin treatment in both human blood as well as human cerebrospinal fluid, an observation with huge clinical

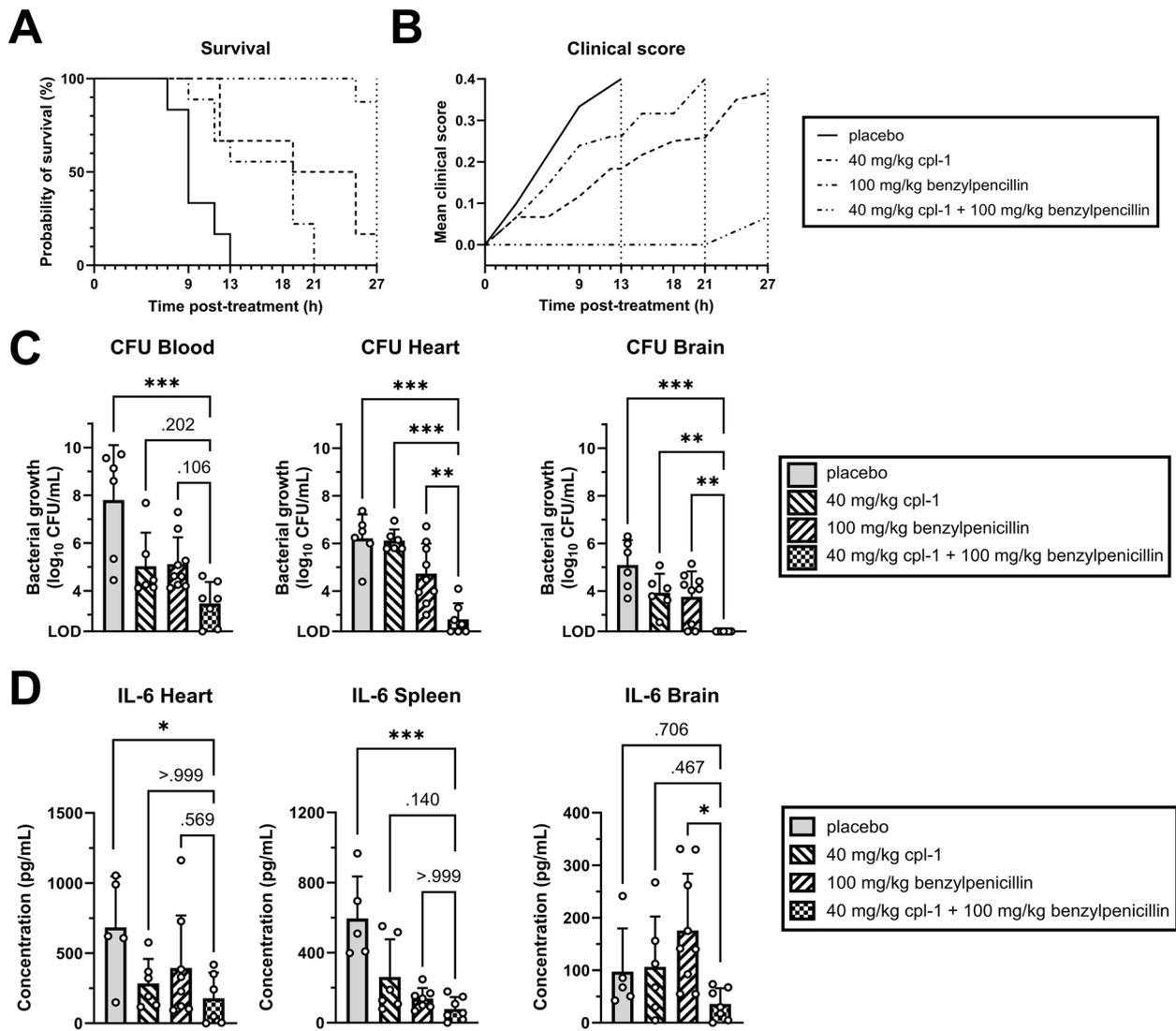


Fig. 5 Administration of cpl-1 endolysin supplemented to penicillin protects mice from penicillin-resistant invasive pneumococcal disease. Kaplan-Meier survival curve (**A**) and mean clinical scores (**B**), assessed according to Karolinska Institutet’s veterinary protocol for rodent physiological and psychological well-being. A clinical score of 0.0 indicates a fully healthy status, while 0.4 indicates the humane endpoint. **C** Bacterial load in the blood, heart, and brain. **D** Levels of IL-6 in the heart, spleen, and brain. In C and D, data were analyzed using one-way ANOVA with Bonferroni post-hoc tests after outlier removal via the ROUT method ($Q = 5\%$); Bars represent the mean \pm standard deviation of biological replicates ($n = 6, 6, 9$ and 7 for placebo, endolysin mono-, antibiotic mono- and combination therapy, respectively; with each dot representing one mouse); LOD indicates the limit of detection (200 CFU/mL); * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$

importance that has not yet been reported. We further characterized the impact of cpl-1 endolysin supplementation to penicillin or erythromycin treatments in resistant pneumococcal strains. Notably, our results revealed that cpl-1 supplementation significantly reduced the MIC of penicillin- and erythromycin-resistant isolates. Moreover, we are the first to demonstrate that this reduction lowers MIC values below clinical resistance breakpoints in a standardized setting as outlined by ESCMID guidelines (EUCAST 2024). We found this interaction

to be synergistic with penicillin, and additive with erythromycin. This marked difference between penicillin and erythromycin can most likely be explained by their different methods of action. Whereas erythromycin blocks the bacterial protein synthesis, penicillin and endolysins both target the pneumococcal cell wall, thus potentially leveraging each other (Vander Elst. et al., 2023, 2024; Djurkovic et al., 2005). Following this observation, our findings strongly suggest that endolysins may be primarily used synergistically with cell wall-targeting antibiotics to

restore antibiotic susceptibility (Vander Elst. et al., 2023, 2024), in line with cell membrane-targeting antibiotics (Vouillamoz, J. et al., 2013). Furthermore, this reduction in MIC has significant pharmacokinetic implications, as the effectiveness of penicillin treatment *in vivo* primarily depends on the duration that drug concentrations remain above the MIC (i.e., $T_{>MIC}$), given that penicillin follows a time-dependent antimicrobial response (Erlendsdottir, H. et al., 2001). The observed synergistic effects between penicillin and the cpl-1 endolysin suggest that lowering the MIC of both agents can substantially extend the $T_{>MIC}$ during antimicrobial treatment. Based on previously reported half-lives of benzylpenicillin and the cpl-1 endolysin in experimental IPD models (Grandgirard et al., 2008; Erlendsdottir, H. et al., 2001), we calculated that penicillin treatment alone would fail after 3 h in our bacteremia-derived meningitis mouse model, whereas supplementing penicillin treatment with cpl-1 endolysin should extend the $T_{>MIC}$ as far as 25 h. Consequently, all mice treated with penicillin alone rapidly developed symptoms and succumbed within 21 h, whereas those receiving the combination therapy indeed survived significantly longer.

Another critical gap that we addressed is whether endolysins should be clinically implemented as a stand-alone treatment, or in combination with standard-of-care antibiotics (Vander Elst, N. 2024; Murray et al., 2021). Both our *in vitro* and *in vivo* experiments clearly demonstrate that endolysin treatment, thus without antibiotic supplementation, could provide therapeutic relief in the case of antibiotic-resistant infections thanks to its strong antibacterial activity; however, due to the short half-life of endolysin, as previously reported (Vander Elst, N. 2024; Grandgirard et al., 2008), pneumococci surviving the endolysin treatment could regrow again. In marked contrast, the combination of endolysin cpl-1 with either penicillin or erythromycin consistently rescued human neuronal cells from antibiotic-resistant infection after only one dosage. We importantly confirmed these findings *in vivo*; in fact, a single cpl-1 endolysin dose together with penicillin significantly reduced penicillin-resistant pneumococci in the blood, heart and spleen, and, remarkably, completely eliminated bacterial presence in the brain. Consequently, mice receiving this combination strategy had a remarkable 89% survival and remained asymptomatic over the course of the infection experiment, with a slight increase of clinical score in the last hours of the experiment due to only one mouse in that group that developed symptoms. The improved survival, symptomatology and bacterial load in the tissues were also confirmed by the reduced inflammation observed in mice that received the combined treatment compared to the groups that received either endolysin or penicillin

alone. Thus, our data strongly suggests that if clinical implementation of endolysin is aimed, which is expected according to the WHO (2023 Antibacterial Agents in Clinical and Preclinical Development an Overview and Analysis n.d.; Vander Elst, N. 2024), their strength lies in a combinatorial approach with antibiotics, irrespective of the resistance levels of the targeted bacteria to the combined antibiotic. This important concept has not previously been reported in the field of Infectious Diseases.

Finally, meningitis is today a dramatic burden in Public Health because of the permanent neurological disabilities caused by neuronal damage inflicted by bacteria in the brain (Lucas et al., 2016; Mohanty, S. et al., 2024). We here provide for the first time clear evidence that endolysin cpl-1 is well capable of penetrating the BBB and successfully reaching the brain upon systemic administration. It was previously reported that intraperitoneal injection of cpl-1 endolysin resulted in a significant reduction of pneumococci in CSF, suggesting that it possesses the ability to cross the BBB, but this could not be distinguished from infection-induced permeability changes or reduced bacterial invasion (Grandgirard et al., 2008). Remarkably, crossing of the BBB was not observed with another endolysin, PlyAZ3a (Valente, L. G. et al., 2022). Given that cpl-1 uniquely binds choline, which is actively transported across the BBB (Inazu, M. 2019), it is plausible that this mechanism facilitates its transport, distinguishing it from other endolysins that primarily bind the bacterial peptidoglycan. Together with our observation of human neuronal cells that were successfully rescued from the cytotoxic effect of antibiotic-resistant infection, our findings suggest that cpl-1 endolysin has the potential to help prevent neurological impairment during meningitis onset, addressing limitations of antibiotics, which struggle to cross the BBB and face growing resistance issues (Nau et al., 2010). However, despite these promising properties, endolysins also have limitations including their short half-life (Grandgirard et al., 2008), potential pro-inflammatory effects due to bacterial antigen release (Murray et al., 2021), induction of anti-drug antibodies (Harhala, M. et al., 2018), and the possibility of resistance development (Bera et al., 2005). These challenges should be carefully considered in future studies to optimize the therapeutic potential of cpl-1 for IPD and pneumococcal meningitis treatment.

In conclusion, this work demonstrates that β -lactam- and macrolide-resistant pneumococci can regain susceptibility to antibiotic treatment through endolysin supplementation. This approach lowers MIC values below clinical resistance breakpoints via synergistic or additive effects with penicillin or erythromycin, respectively. *In vivo*, this restored susceptibility results in significant systemic bacterial load reductions, and to the complete clearance of bacterial

presence in the brain; a strong antibacterial effect that is accompanied by a reduced systemic and neuroinflammation and that, overall, contributes to an asymptomatic course of infection and a significantly increased probability of survival. Moreover, the ability of endolysins to effectively cross the BBB, protect human neuronal cells from the cytotoxic effects of antibiotic-resistant infections, and completely eradicate antibiotic-resistant pneumococci in the brain, highlights its remarkable potential as an adjunct to antibiotic treatment not only for invasive diseases, such as bacteremia, but also for pneumococcal meningitis.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s10020-025-01226-1>.

Supplementary Material 1.

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Authors' contributions

Conception or design of the work: NV and FI; data collection: NV, KF, LK and LM; data analysis and interpretation: NV, KF, LK, LM and FI; drafting the article: NV and FI; critical revision of the article: NV, KF, LK, LM and FI. All authors have read and agreed to the current version of the manuscript.

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Data availability

All data generated or analyzed during this study are included in the published article and are available on the Mendeley Dataset DOI: <https://doi.org/10.17632/9b55khv693.1>.

Declarations

Ethics approval and consent to participate

Animal experiments were performed in accordance with Swedish legislation (Svenska jordbruksverket), and ethical approval (permit number 18965–2021).

Competing interests

NV declares issued and pending patent applications with regards to endolysin technology.

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